STATE OF NEW YORK   )
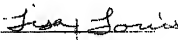   )   ss
COUNTY OF NEW YORK   )
)

AFFIDAVIT OF TRANSLATION

Lisa Louis, being duly sworn, deposes and says: I am a professional translator of the Japanese language with many years of experience; I have prepared the attached English translation of Japanese Patent Application JP 2004-045111, as provided in Priority Certificate No. 2004-3097940 that was filed in PCT/JP2004/013536, and which to the best of my knowledge and belief is true and accurate.

_____
            Lisa Louis
_____
          July 28, 2009

This is to certify that the items noted in the attached documents are the same as the items noted in the following application.

Date of Application: February 20, 2004

Application No.:   App. No. 2004-045111
[ST. 10/C]:   [JP 2004-045111]

Applicant(s):   Immuno-Biological Laboratories, Co., Ltd.

PRIORITY DOCUMENT
Submitted or Transmitted in Compliance with Rule 17.1 (a) or (b)

October 29, 2004

Commissioner, Japan Patent Office   **Hiroshi Ogawa**   [seal:] Patent Office Commissioner

[Document Title]	Patent Application
[Reference Number]	0310131
[Filing Date]	February 20, 2004
[Address]	Commissioner of the Patent Office
[Inventor]	
[Domicile or Address]	Gunma University, Faculty of Medicine, School of Health Sciences
	1022-13 Shimo Shinden-machi, Maebashi, Gunma Prefecture
[Name]	Haruyasu Yamaguchi
[Inventor]	
[Domicile or Address]	Immuno-Biological Laboratories, Co., Ltd.
	1091-1 Naka Aza Higashida, Fujioka, Gunma Prefecture
[Name]	Noriaki Kinoshita
[Inventor]	
[Domicile or Address]	Immuno-Biological Laboratories, Co., Ltd.
	1091-1 Naka Aza Higashida, Fujioka, Gunma Prefecture
[Name]	Masahiro Maeda
[Inventor]	
[Domicile or Address]	Immuno-Biological Laboratories, Co., Ltd.
	1091-1 Naka Aza Higashida, Fujioka, Gunma Prefecture
[Name]	Yuko Horikoshi
[Patent Applicant]	
[Identification Number]	399032282
[Name or Title]	Immuno-Biological Laboratories, Co., Ltd.
[Agent]	
[Identification Number]	100086324
[Patent Attorney]	
[Name or Title]	Nobuo Ono
[Appointed Agent]	
[Identification Number]	100125748
[Patent Attorney]	
[Name or Title]	Tokuaki Takahashi
[Indication of Fees]	
[Prepayment Register Number]	007375
[Amount of Payment]	¥21,000
[List of Documents Submitted]	
[Document Title]	Claims 1
[Document Title]	Specification 1
[Document Title]	Drawings 1
[Document Title]	Abstract 1
[Comprehensive Letter of Attorney Number]	9906100
[Comprehensive Letter of Attorney Number]	0305546

[Document Title] Claims
[Claim 1]
     A monoclonal antibody which can recognize an N-terminus peptide of an amyloid beta, but does not recognize an amyloid beta precursor protein.
[Claim 2]
     A monoclonal antibody according to claim 1, wherein the N-terminus peptide of the amyloid beta is a peptide shown by the following amino acid sequence (a).
     (a) DAEFRHDSGYEVHHQK (Sequence ID No. 1)
[Claim 3]
     A monoclonal antibody according to claim 1, wherein the N-terminus peptide of the amyloid beta is a peptide shown by the following amino acid sequence (b).
     (b) DAEFR (Sequence ID No. 2)
[Claim 4]
     A monoclonal antibody according to claim 1, which is obtained by immunizing an animal with a bound substance of the N-terminus peptide of the amyloid beta and a biological high molecular compound as a first antigen, subsequently immunizing the thus-immunized animal with a bound substance of another N-terminus peptide of the amyloid beta, which is comparatively shorter than the peptide used for the first antigen, and a biological high molecular compound as a second antigen, and collecting the antibody from the animal.
[Claim 5]
     A monoclonal antibody according to claim 1, which is obtained by immunizing an animal with a bound substance of a peptide shown by the amino acid sequence (a) and a biological high molecular compound as a first antigen, subsequently immunizing the thus-immunized animal with a bound substance of a peptide shown by the amino acid sequence (b) and a biological high molecular compound as a second antigen, and collecting the antibody from the animal.
     (a) DAEFRHDSGYEVHHQK (Sequence ID No. 1)
     (b) DAEFR (Sequence ID No. 2)
[Claim 6]
     A kit for assaying amyloid beta comprising a first reagent containing an antibody which can recognize the N-terminus peptide of amyloid beta, but does not recognize amyloid beta precursor proteins, and a second reagent containing an antibody which can recognize amyloid beta (1-40) or amyloid beta (1-42).
[Claim 7]
     A kit for assaying amyloid beta according to claim 6, wherein the antibody recognizing an amyloid beta (1-40) or amyloid beta (1-42) is an antibody recognizing the C-terminus peptide of the amyloid beta.
[Claim 8]
     A kit for assaying amyloid beta according to claim 7, wherein the C-terminus peptide of amyloid beta is a peptide shown by the following amino acid sequence (c).
     (c) MVGGVV (Sequence ID No. 3)
[Claim 9]
     A kit for assaying amyloid beta according to claim 8, wherein the kit is for assaying amyloid beta (1-40).
[Claim 10]
     A kit for assaying amyloid beta according to claim 7, wherein the C-terminus peptide of the amyloid beta is a peptide shown by the following amino acid sequence (d).
     (d) GVVIA (Sequence ID No. 4)
[Claim 11]
     A kit for assaying amyloid beta according to claim 10, wherein the kit is for assaying amyloid beta (1-42).
[Claim 12]
     A method for assaying amyloid beta characterized by having an antibody which can recognize the N-terminus peptide of an amyloid beta, but does not recognize amyloid beta precursor proteins, and an antibody which can recognize

amyloid beta (1-40) or amyloid beta (1-42) react with an amyloid beta in a sample to be assayed.

[Claim 13]

A method for assaying amyloid beta according to claim 12, wherein the antibody recognizing an amyloid beta (1-40) or amyloid beta (1-42) is an antibody recognizing the C-terminus peptide of the amyloid beta.

[Claim 14]

A method for preparing a monoclonal antibody of claim 1 characterized by immunizing an animal with a bound substance of the N-terminus peptide of the amyloid beta and a biological high molecular compound as a first antigen, subsequently immunizing the thus-immunized animal with a bound substance of another N-terminus peptide of the amyloid beta, which is comparatively shorter than the peptide used for the first antigen, and a biological high molecular compound as a second antigen, and collecting the antibody from the animal.

[Document Title] Specification
[Title of the Invention] Monoclonal Antibody and Use Thereof
[Technical Field]
[0001]
        The present invention relates to a monoclonal antibody which can recognize an amyloid beta, and more specifically, relates to a monoclonal antibody which can precisely assay amyloid beta (1-40) and amyloid beta (1-42) which completely possess the entire length, and the use thereof.
[Background Art]
[0002]
        An amyloid beta is a peptide consisting of 40 or 42 amino acids and is generated from an amyloid beta precursor protein (APP) cleaved by beta-secretase and gamma-secretase. The amyloid beta with 40 amino acids is referred to as amyloid beta (1-40) and the amyloid beta with 42 amino acids is referred to as amyloid beta (1-42). The respective amino acid sequences thereof are shown below.
        Amyloid beta (1-40) (Amino acid sequence No. 5):
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV
        Amyloid beta (1-42) (Amino acid sequence No. 6):
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
[0003]
        Of these amyloid beta, the amyloid beta (1-40) is said to be a peptide cleaved via a common metabolic pathway and to have weak toxicity. On the other hand, the amyloid beta (1-42) is said to be insoluble, have strong toxicity, easily agglomerate into a fibrous form, and be accumulated in the brain and cause Alzheimer's disease.
[0004]
        Therefore, assaying each amyloid beta peptide is very important in diagnosing Alzheimer's disease and investigating the mechanism of the disease occurrence.
[0005]
        Heretofore, an amyloid beta antibody has been used for assaying an amyloid beta (for example, Patent Reference 1). However, since the amyloid beta antibody described in Patent Reference 1 has an epitope in the 3-8 site of the amino acid sequence of the amyloid beta, an amyloid beta assay kit using this detects amyloid beta precursor proteins (APP) as well as amyloid beta (1-40) and amyloid beta (1-42) possessing the entire length of the amino acid sequence. A presently commercially available amyloid beta antibody (6E10 manufactured by Signet Laboratories, Inc.) also recognizes APP.
[0006]
        [Patent Reference 1]   International Publication WO 1994/017197 pamphlet
[Disclosure of the Invention]
[Problems to be Solved by the Invention]
[0007]
        Therefore, an object of the invention is to provide a system for precisely assaying amyloid beta (1-40) and amyloid beta (1-42) which completely possess the entire length.
[Means for Solving the Problems]
[0008]
        As a result of extensive studies in order to provide a system for precisely assaying amyloid beta (1-40) and amyloid beta (1-42) which completely possess the entire length, the inventors have found that it is possible to precisely assay amyloid beta (1-40) and amyloid beta (1-42) (hereinafter referred to from time to time collectively as "amyloids beta") by using a specific monoclonal antibody which can recognize an amyloid beta, but does not recognize amyloid beta precursor protein or the like.
[0009]

Specifically, the present invention provides a monoclonal antibody which can recognize the N-terminus peptide of amyloid beta, but does not recognize amyloid beta precursor proteins.

[0010]
        Also, the present invention also provides a kit for assaying amyloid beta comprising a first reagent containing an antibody which can recognize the N-terminus peptide of amyloids beta, but does not recognize amyloid beta precursor proteins and a second reagent containing an antibody which can recognize amyloid beta (1-40) or amyloid beta (1-42).

[0011]
        Furthermore, the present invention provides a method for assaying amyloids beta characterized by having an antibody which can recognize the N-terminus peptide of an amyloid beta, but does not recognize amyloid beta precursor proteins, and an antibody which can recognize amyloid beta (1-40) or amyloid beta (1-42) react with an amyloid beta in a sample to be assayed.

[0012]
        Yet further, the present invention provides a method for producing the above monoclonal antibody characterized by immunizing an animal with a bound substance of the N-terminus peptide of an amyloid beta and a biological high molecular compound as a first antigen and subsequently immunizing the thus-immunized animal with a bound substance of another N-terminus peptide of an amyloid beta, which is comparatively shorter than the peptide used for the first antigen, and a biological high molecular compound as a second antigen, and collecting the antibody from the animal.

[Effect of the Invention]

[0013]
        The monoclonal antibody of the present invention recognizes the N-terminus peptide of an amyloid beta, but does not recognize an amyloid beta precursor protein.

[0014]
        Therefore, the use of this antibody makes it possible to construct a system for precisely assaying amyloid beta (1-40) and amyloid beta (1-42) which completely possess the entire length, which has been difficult heretofore.

[Best Mode for Carrying Out the Invention]

[0015]
        It is difficult to obtain the monoclonal antibody of the present invention which can recognize the N-terminus peptide of amyloid beta, but does not recognize amyloid beta precursor proteins (hereinafter referred to as "N-terminus antibody") by immunizing an animal with an N-terminus peptide of an amyloid beta using a conventional technique. This is because the amino acid sequence of the amyloid beta is completely identical with a part of the amyloid beta precursor proteins, so it is difficult to obtain an antibody that can distinguish these two items.

[0016]
        Therefore, in order to obtain the N-terminus antibody of the present invention, it is necessary to immunize an animal with a bound substance of the N-terminus peptide of an amyloid beta and a biological high molecular compound as a first antigen and to subsequently immunize the thus-immunized animal with a bound substance of another N-terminus peptide of an amyloid beta, which is comparatively shorter than the peptide used for the first antigen, and a biological high molecular compound as a second antigen, and collect the antibody from the animal.

[0017]
        The method for preparing the N-terminus antibody of the present invention using the aforementioned first antigen and second antigen will be described below.

[0018]
        The N-terminus peptide of the amyloid beta used for the first antigen is a peptide consisting of a continuous amino acid sequence from the N-terminus to the C-terminus of the amino acid sequence of amyloid beta (1-40) or amyloid beta (1-42) (Sequence ID No. 5 or Sequence ID No. 6), preferably a peptide consisting of amino acid sequence 1 to 28 of the amyloid beta, and particularly preferably a peptide containing amino acid sequence 1 to 16 of the amyloid beta which is shown by the following amino acid sequence (a).

[0019]
        (a) DAEFRHDSGYEVHHQK (SEQ ID No. 1)

[0020]
The peptide consisting of the above amino acid sequence can be obtained by various methods without any specific limitations. For example, it may be synthesized by a method known in the art, or a synthetic product commercially available from Synpep Corporation and TANA Laboratories, USA, etc. may be purchased.

[0021]
The peptide consisting of the above amino acid sequence is next bound with a biological high molecular compound, and this is used as a first antigen. In this case, binding is preferably done after attaching cysteine (C) to the amino acid at the C-terminus side of the peptide.

[0022]
Examples of the biological high molecular compound bound with the peptide include keyhole limpet hemocyanin (hereafter referred to as KLH), ovalbumin (hereafter referred to as OVA), bovine serum albumin (hereafter referred to as BSA), rabbit serum albumin (hereafter referred to as RSA), thyroglobulin, and the like, and of these, KLH and thyroglobulin are preferable.

[0023]
The aforementioned peptide can be bound with the biological high molecular compound by a known method such as a mixed acid anhydride method (B. F. Erlanger, et al., J. Biol. Chem., 234, 1090–1094 (1954)), an activated ester method (A. E. KARU, et al., J. Agric. Food Chem., 42, 301–309 (1994)), and the like.

[0024]
A mixed acid anhydride used in the mixed acid anhydride method is obtained by subjecting the aforementioned peptide to a common Schotten-Baumann reaction, and this is reacted with the biological high molecular compound to create the target bound substance of the peptide and biological high molecular compound. Examples of the haloformate ester used in this mixed acid anhydride method include methyl chloroformate, methyl bromoformate, ethyl chloroformate, ethyl bromoformate, and isobutyl chloroformate. The ratio of the peptide, haloformate ester, and high molecular compound used in this method can be selected as appropriate from a wide range.

[0025]
The Schotten-Baumann reaction is carried out in the presence of a basic compound. As the basic compound used for the reaction, compounds commonly used in the Schotten-Baumann reaction, for example, organic bases such as triethylamine, trimethylamine, pyridine, dimethylaniline, N-methylmorpholine, diazabicyclononene (DBN), diazabicycloundecene (DBU), and diazabicyclooctane (DABCO), and inorganic bases such as potassium carbonate, sodium carbonate, potassium hydrogencarbonate, and sodium hydrogencarbonate and the like can be used.

[0026]
Also, the reaction is usually carried out at a temperature from -20 °C to 100 °C, and preferably from 0 °C to 50 °C, for 5 minutes to 10 hours, and preferably 5 minutes to 2 hours.

[0027]
The reaction of the obtained mixed acid anhydride and the biological high molecular compound is carried out at a temperature usually from -20 °C to 150 °C, and preferably from 0 °C to 100 °C, for 5 minutes to 10 hours, and preferably 5 minutes to 5 hours. The mixed acid anhydride method is typically carried out in a solvent, and any solvent commonly used in the mixed acid anhydride method can be used, with specific examples including halogenated hydrocarbons such as dichloromethane, chloroform, and dichloroethane; aromatic hydrocarbons such as benzene, toluene, and xylene; ethers such as diethyl ether, dioxane, tetrahydrofuran, and dimethoxyethane; esters such as methyl acetate and ethyl acetate; and aprotic polar solvents such as N,N-dimethylformamide, dimethyl sulfoxide, and hexamethylphosphate triamide, and the like.

[0028]
On the other hand, the activated ester method can be carried out as follows. First, the peptide is dissolved in an organic solvent and reacted with N-hydroxysuccinimide in the presence of a coupling agent

to produce an N-hydroxysuccinate imide activated ester.

[0029]

As the coupling agent used in this reaction, any coupling agents commonly used in a condensation reaction can be used, with examples including dicyclohexylcarbodiimide, carbonyldiumidazole, water-soluble carbodiimide and the like. As an organic solvent, for example, N,N-dimethylformamide (DMF), dimethyl sulfoxide, dioxane, and the like can be used. The molar ratio of the peptide and the coupling agent such as N-hydroxysuccinate imide used for the reaction is preferably from 1:10 to 10:1, and most preferably 1:1. The reaction is carried out at a temperature usually from 0 °C to 50 °C, and preferably from 22 °C to 27 °C, for 5 minutes to 24 hours, and preferably 1 to 2 hours. A reaction temperature not lower than the melting point, but not higher than the boiling point of the reactants can be used.

[0030]

After the coupling reaction, the reaction solution is added to and reacted with a solution in which the biological high molecular compound is dissolved to produce, for example, when the biological high molecular compound has a free amino group, an acid amide bond between the amino group and the carboxyl group of the above peptide. The reaction is carried out at a temperature usually from 0 °C to 60 °C, preferably from 5 °C to 40 °C, and more preferably from 22 °C to 27 °C, for 5 minutes to 24 hours, preferably 1 to 16 hours, and more preferably 1 to 2 hours.

[0031]

The reaction products obtained by any of the above methods are purified by dialysis, desalting column, and the like to obtain a bound substance of the peptide and biological high molecular compound (hereinafter referred to from time to time as "bound substance 1").

[0032]

The N-terminus peptide of the amyloid beta comparatively shorter than the peptide used for the first antigen, which is used for the second antigen, is a peptide consisting of a continuous amino acid sequence from the N-terminus to the C-terminus of the amino acid sequence of amyloid beta similar to the peptide used for the first antigen. There are no specific limitations to such a peptide as long as the length is shorter than the length of the peptide used for the first antigen, with a peptide for which the length is shorter by 3 to 11 amino acids, preferably 5 to 11 amino acids, than the peptide used for the first antigen being preferable. As such a peptide, a peptide consisting of amino acid sequence 1-13 of amyloid beta is preferable, with a more preferable peptide being that consisting of amino acid sequence 1-11 of amyloid beta. A peptide shown by the following amino acid sequence (b) which contains amino acid sequence 1-5 of the amyloid beta is particularly preferable.

[0033]

(b) DAEFR (Sequence ID No. 2)

[0034]

The above peptide can produce a bound substance with a biological high molecular compound (hereinafter referred to from time to time as "bound substance 2") in the same manner as the method for producing the bound substance 1.

[0035]

Next, the method for preparing the N-terminus antibody using the bound substance 1 and the bound substance 2 obtained in this manner as antigens will be described. Note that any known method for preparing the antibody, such as a method described in, for example, Immunobiochemical Research Method in the Sequel Biochemical Experiment Lecture (edited by the Japanese Biochemical Society) can be appropriately used, except for the use of the bound substance 1 and the bound substance 2.

[0036]

In order to prepare the N-terminus antibody of the present invention using the above bound substances, an animal is first immunized using the bound substance 1 as a first antigen, the immunized animal is subsequently immunized using the bound substance 2 as a second antigen, and the resulting antibody is collected from the animal.

[0037]

As a specific immunization method, first, the bound substance 1 is dissolved in a sodium phosphate buffer solution (PBS), this is mixed with a Freund's complete adjuvant, incomplete adjuvant, or an assisting agent such as alum, and an animal is immunized. with this as a first antigen.

[0038]

As the animal to be immunized, any animals commonly used in the art, for example,

mammals such as a mouse, rat, rabbit, goat, or horse can be used. The method of administering the immunogen for the immunization may be subcutaneous injection, intraperitoneal injection, intravenous injection, intradermal injection, or intramuscular injection, with subcutaneous injection and intraperitoneal injection being preferable. For immunization, the immunogen is administered one time or a plurality of times at an appropriate interval, preferably a plurality of times at an interval of one to five weeks.

[0039]

Subsequently, the animal immunized with the first antigen in this manner is immunized with a second antigen, which is prepared using the bound substance 2 in the same manner as in the first antigen. In the same manner as in immunizing with the first antigen, the immunogen is administered one time or a plurality of times at an appropriate interval, preferably a plurality of times at an interval of one to five weeks.

[0040]

Finally, immunocytes obtained from the immunized animal is fused with myeloma cells according to a conventional method to obtain a hybridoma. The monoclonal antibody to the N-terminus of the amyloid beta can be obtained by collecting the antibody from the hybridoma culture.

[0041]

The N-terminus antibody of the present invention obtained in this way can be used for immunoassay by labeling or immobilizing if necessary.

[0042]

For labeling, a labeling substance such as enzymes (horseradish peroxidase (HRP), alkaline phosphatase, etc.), fluorescent substances (fluoresceine isocyanate, rhodamine, etc.), radioactive materials (^{32}P, ^{125}I, etc.), chemoluminescent substances, and the like is bound with the N-terminus antibody. In order to immobilize the N-terminus antibody, the antibody is bound with an appropriate immobilizing medium. Any immobilizing media conventionally used in the immunochemical measuring method, for example, a plate such as a 96-well microtiter plate made from polystyrene, an amino group-bonded microtiter plate, etc., and various types of beads can be used. In order to immobilize the N-terminus antibody, a buffer solution containing the antibody, for example, is added to a carrier and incubated.

[0043]

The N-terminus antibody of the present invention can be used for precisely assaying amyloid beta particularly by combining with an antibody that recognizes amyloid beta (1-40) or amyloid beta (1-42).

[0044]

As the antibody recognizing amyloid beta (1-40) or amyloid beta (1-42) to be combined with the N-terminus antibody of the present invention, a polyclonal antibody or a monoclonal antibody that can be obtained using a part or whole of the amino acid sequence of the amyloid beta (1-40) or amyloid beta (1-42) as an antigen by a conventional method, for example, can be used. With the present invention, of these antibodies, an antibody recognizing the C-terminus peptide of the amyloid beta (hereinafter referred to as "C-terminus antibody") is preferable due to the accuracy of the assay.

[0045]

The C-terminus antibody is not specifically limited so long as the antibody can recognize the C-terminus peptide of the amyloid beta (1-40) or amyloid beta (1-42) to be assayed. It is, however, desirable that the antibody does not recognize an amyloid beta other than the amyloid beta (1-40) or amyloid beta (1-42) to be assayed, such as an amyloid beta (1-43), for example.

[0046]

Specifically, the C-terminus antibody recognizing the amyloid beta (1-40) (hereinafter referred to as "C-terminus antibody 1") can be obtained by a conventional method using a peptide (C-terminus peptide) consisting of a continuous amino acid sequence from the C-terminus to the N-terminus of the amyloid beta (1-40) (Sequence ID No. 5) as an antigen. More specifically, C-terminus peptide used as an antigen is preferably a peptide consisting of amino acid sequence 18-40 of amyloid beta (1-40), and particularly preferably amino acid sequence 35-40 of amyloid beta (1-40) shown by the following amino acid sequence (c).

[0047]

(c) MVGGVV (Sequence ID No. 3)

[0048]

In the same way, the C-terminus antibody recognizing the amyloid beta (1-42) (hereinafter referred to as "C-terminus antibody 2") can be obtained by a conventional method using a peptide consisting of a continuous amino acid sequence from the C-terminus to the N-terminus of the amyloid beta (1-42) (Sequence ID No. 6) as an antigen. More specifically, the peptide used as an antigen is preferably a peptide consisting of amino acid sequence 18-42 of amyloid beta (1-42), and particularly preferably amino acid sequence 38-42 of amyloid beta (1-42) shown by the following amino acid sequence (d).

[0049]

(d) GVVIA (Sequence ID No. 4)

[0050]

In addition, when preparing the C-terminus antibody using the above peptide, a bound substance of the peptide with a biological high molecular compound may be used as an antigen in the same way as in the preparation of the N-terminus antibody. In this bound substance of the peptide with a biological high molecular compound, the peptide is preferably bound after attaching lysine-cysteine (KC), for example, to the amino acid of the N-terminus side.

[0051]

As the antibody recognizing amyloid beta (1-40) or amyloid beta (1-42), the following antibodies commercially available from Immuno-Biological Laboratories Co., Ltd. can be used.

Anti-Human Amyloid beta (35-40) (1A10) Mouse IgG MoAb (Product number: 10047)

Anti-Human Amyloid beta (1-40) Rabbit IgG Affinity Purify (Product number: 18580)

Anti-Human Amyloid beta (1-42) Rabbit IgG Affinity Purify (Product number: 18582)

[0052]

Amyloid beta (1-40) or amyloid beta (1-42) can be precisely assayed by using the N-terminus antibody of the present invention and the antibody recognizing the amyloid beta (1-40) or amyloid beta (1-42). As specific assay methods, various methods currently used in common immunochemical measuring such as radioisotope immunoassay (RIA method), ELISA method (E. Engvall et al. (1980), Methods in Enzymol., 70, 419–439), fluorescent antibody technique, plaque method, spot method, condensation method, Ouchterlony, immunochromatography, and the like ("Hybridoma method and monoclonal antibody" R&D Planning Inc., pp 30–53, Mar. 5, 1982) can be used.

[0053]

Although an appropriate method can be selected from these assay methods taking various factors into consideration, the ELISA method is preferable due to the high sensitivity, ease of assaying, and the like. A specific procedure of assaying amyloid beta will be described taking a sandwich method, which is one of the ELISA methods, as an example.

[0054]

First, as a step (A), an antibody recognizing amyloid beta (1-40) or amyloid beta (1-42) is immobilized on a carrier. Next, as a step (B), the surface of the carrier on which the antibody is not immobilized is blocked with a substance irrelevant to amyloid beta, for example, a protein. As a step (C), test samples containing amyloid beta at different concentrations are added to produce a complex of amyloid beta and the antibody. Then, as a step (D), the labeled N-terminus antibody of the present invention is added to combine the N-terminus antibody with the complex of amyloid beta and the antibody. Finally, as a step (E), the amount of the substance used as a label is measured to determine the amount of amyloid beta in the sample using a previously prepared calibration curve. Although the measurement is possible when the antibody used in step (A) and the antibody used in step (D) are reversed, immobilization of the antibody recognizing amyloid beta (1-40) or amyloid beta (1-42) is more preferable from the viewpoint of detection sensitivity.

[0055]

There are no specific limitations to the carrier used for immobilizing the antibody recognizing amyloid beta (1-40) or amyloid beta (1-42) in step (A). Any carriers commonly used in immunochemical assay can be used. Examples include a 96-well microtiter plate made of polystyrene or an amino group bound-type microtiter plate.

In order to immobilize the above antibody, a buffer solution containing the antibody, for example, is added to a carrier and incubated. As the buffer solution, known buffer solutions can be used, with examples including a 10 mM PBS. Although the concentration of the antibody in the buffer solution can be selected from a wide range, usually a range from about 0.01 to 100 μg/ml, and preferably from 0.1 to 20 μg/ml is used. When a 96-well microtiter plate is used as a carrier, the volume per well is 300 μl or less, and preferably about 20 to 150 μl. There are also no specific limitations to the incubation conditions, but usually, incubation overnight at about 4 °C is appropriate.

[0056]
        Blocking in step (B) is to inhibit amyloid beta in a test sample from being adsorbed in the carrier, on which the antibody has been immobilized in step (A), irrespective of the antigen-antibody reaction. As a blocking agent, BSA, a skim milk solution, and commercially-available blocking agents such as "Block Ace" manufactured by Dainippon Pharmaceutical Co., Ltd. (code No.UK-25B) can be used, for example. Although not limited, a specific blocking method comprises, for example, adding an appropriate amount of Block Ace to a portion in which an antigen has been immobilized and incubating overnight at about 4 °C, followed by washing with a buffer solution.

[0057]
        Furthermore, in step (C), the test sample containing an amyloid beta is caused to come in contact with an immobilized antibody and the amyloid beta is captured with the immobilized antibody to produce a complex of the immobilized antibody and the amyloid beta. The conditions for producing the complex is not limited, but the reaction can be conducted at about 4 °C to 37 °C for about 1 hour to overnight. After the reaction, the carrier is preferably washed with a buffer solution to remove unreacted proteins and the like. As the buffer solution used for this reaction, a 10 mM PBS (pH 7.2) containing 0.05% (v/v) Tween 20 composition is preferable.

[0058]
        Furthermore, in step (D), a labeled antibody which recognizes a different epitope of the amyloid beta captured by the immobilized antibody is added to produce a complex of the immobilized antibody, amyloid beta, and the labeled antibody. After the reaction, the carrier is preferably washed with a buffer solution to remove unreacted proteins and the like. The same buffer solution as that described above is used for this reaction. The amount of the labeled antibody used in step (D) is about 5,000 to 10,000 times that of the immobilized antibody, preferably an amount diluted to a concentration in which the ultimate absorbance is 1.5-2.0. A buffer solution can be used for dilution and the reaction is carried out at about 4 °C to 37 °C for about 1 hour, for example. After the reaction, the complex substance is preferably washed with the buffer solution. The complex of the immobilized antibody, amyloid beta, and the labeled antibody can be formed by the above reaction.

[0059]
        In step (E), a coloring substrate solution reactive with the labeling substance in the complex of the immobilized antibody, amyloid beta, and the labeled antibody is added and absorbance is measured to calculate the amount of amyloid beta in reference to a calibration curve.

[0060]
        When peroxidase, which is an enzyme, is used for labeling the antibody, a coloring substrate solution containing hydrogen peroxide and 3,3',5,5'-tetramethylbenzene (hereinafter referred to as "TMB") can be used, for example. Although not limited, the coloring reaction can be carried out by adding the coloring substrate solution, reacting at about 25 °C for about 30 minutes, and adding a 1-2 N sulfuric acid aqueous solution to terminate the enzyme reaction. When TMB is used, the coloration is determined by measuring absorbance at 450 nm. When alkaline phosphatase enzyme is used as a labeling substance, on the other hand, p-nitrophenylphosphoric acid is used as a coloring substrate, a 2N NaOH solution is added to terminate the enzyme reaction, and the absorbance at 415 nm is measured. The concentration of amyloid beta in a sample can be calculated by using a calibration curve previously prepared using the absorbance of a reaction solution to which amyloid beta with a known concentration is added.

[0061]

In order to assay amyloid beta according to the method described above, a kit for assaying amyloid beta comprising a first reagent containing the N-terminus antibody of the present invention and a second reagent containing an antibody which can recognize amyloid beta (1-40) or amyloid beta (1-42) is preferably used. Such a kit is hereinafter referred to as "kit of the present invention."

[0062]

The kit of the present invention can be prepared according to a conventional method. Specifically, the N-terminus antibody of the present invention or an antibody which recognizes amyloid beta (1-40) or amyloid beta (1-42) as a labeled antibody, and a buffer solution for dilution, a standard substance, a buffer solution for a substrate, a termination solution, washing fluid, and the like are combined.

[0063]

An amyloid beta (1-40) or amyloid beta (1-42) completely possessing the entire length in samples such as plasma and serum can be precisely assayed using the above measuring method and the assay kit.

[Embodiments]

[0064]

The present invention will be described in more detail by embodiments, which should not be construed as limiting the present invention. Any person having an ordinary skill in the art can easily modify or alter the inventions based on the description in this specification. Such modifications and alterations are included in the technological scope of the present invention.

[0065]

Embodiment 1

Procurement of N-terminus Peptide and C-terminus Peptide of Amyloid beta:

N-terminus peptide and C-terminus peptide of amyloid beta purified by HPLC were purchased from Synpep Corporation and TANA Laboratories, USA. The amino acid sequences of these peptides are shown in (e), (f), (g), and (h) below. Note that the peptide (e) has cysteine (C) attached to amino acid sequence 1-16 (Sequence ID No. 1) of amyloid beta, the peptide (f) has C attached to amino acid sequence 1-5 (Sequence ID No. 2) of amyloid beta, the peptide (g) has KC attached to amino acid sequence 35-40 (Sequence ID No. 3) of amyloid beta (1-40), and the peptide (h) has lysine-cysteine (KC) attached to amino acid sequence 38-42 (Sequence ID No. 4) of amyloid beta (1-42).

[0066]

(e) DAEFRHDSGYEVHHQKC

(f) DAEFRC

(g) KCMVGGVV

(h) KCGVVIA

[0067]

Embodiment 2

Preparation of Antigen for Immunization:

Bound substances of each of the above peptides and thyroglobulin were prepared by the EMCS (N-(6-Maleimidocaproyloxy)-succinimide) method as follows. The molar ratio of the thyroglobulin, peptide, and EMCS used for preparing the bound substances was 1:300:400.

[0068]

First, 4 mg of each peptide of embodiment 1 was dissolved in about 1 ml of distilled water. Meanwhile, a solution of 5 mg of thyroglobulin in 1 ml of a 0.01 M phosphoric acid buffer (pH 7.0) and a solution of 80 µg/µl EMCS dissolved in dimethylformamide were mixed in an amount to obtain a thyroglobulin-EMCS complex solution containing the thyroglobulin and EMCS at the above molar ratio. This complex solution was divided into four portions, to each of which was added each peptide solution in an amount to make the above molar ratio, thereby obtaining a solution of a bound substance of the peptide crosslinked by EMCS and the thyroglobulin.

[0069]

This bound substance solution was dialyzed using PBS to obtain a solution with a bound substance concentration of 10 µg/µl. The bound substances of each peptide and thyroglobulin obtained in this manner were used as antigens for immunization in the following examples.

[0070]

Embodiment 3

Preparation of Antibody Recognizing C-terminus Peptide of Amyloid beta (1-40):

A mouse was immunized using the bound substance of the peptide (g) and thyroglobulin obtained in embodiment 2 as an antigen for immunization by administering 50 µl (50 µg) of the bound substance solution at an interval of one or two weeks. The antigen was mixed with a complete Freund's adjuvant only in initial immunization and mixed with an incomplete Freund's adjuvant in the subsequent immunization. Spleen monocyte cells of the immunized mouse and a fusion partner, X63-Ag8-653, were subjected to polyethylene glycol-mediated cell fusion and hybridomas were selected using the method described in J. Immunol. 146:3721–3728. In selecting the hybridomas, cells that react with immobilized peptide (g), but appear not reactive with peptide (h) were selected.

[0071]

The cells selected in this manner were cultured in a serum-free GIT culture medium (manufactured by Wako Pure Chemical Industries, Ltd.) to produce antibodies until 80% of the cells were extinct. Next, after removing the cells from the culture medium by centrifugation (1,000 rpm, 15 min), the resultant fluid was brought to a 50% saturated state with ammonium sulfate, allowed to stand still at 4 °C overnight, and the resultant precipitate was collected by centrifugation (1,000 rpm, 30 min). Furthermore, after dissolving the precipitate in a twice-diluted binding buffer (manufactured by Protein AMAPS IIkit), IgG was adsorbed in Protein A column (manufactured by Pharmacia Amersham). The product was purified by PBS dialysis overnight to obtain an antibody recognizing the C-terminus peptide of amyloid beta (1-40). This antibody was named 1A10.

[0072]

Embodiment 4

Confirmation of Specificity of 1A10 Antibody by Western Blotting:

In order to confirm that the 1A10 antibody obtained in embodiment 3 recognizes the C-terminus peptide of the amyloid beta (1-40), the 1A10 antibody was subjected to western blotting against each peptide of amyloid beta (1-40), amyloid beta (1-42), and amyloid beta (1-43) according to a common western blotting method (for example, "Molecular Biology Basic Experimentation" Nankodo Co., Ltd.).

[0073]

The results of western blotting are shown in FIG. 1. It was confirmed that the monoclonal antibody 1A10 does not react with amyloid beta (1-42) and amyloid beta (1-43), but recognizes only amyloid beta (1-40).

[0074]

Embodiment 5

Preparation of Antibody Recognizing C-terminus Peptide of Amyloid beta (1-42):

A mouse was immunized in the same manner as in embodiment 3 by using the bound substance of the peptide (h) and thyroglobulin obtained in embodiment 2 as an antigen for immunization. Spleen monocyte cells of the immunized mouse and a fusion partner, X63-Ag8-653, were subjected to polyethylene glycol-mediated cell fusion and hybridomas were selected using the method described in J. Immunol. 146:3721–3728. In selecting the hybridomas, cells that react with immobilized peptide (h), but appear not reactive with peptide (g) were selected.

[0075]

The cells selected by the above procedure were purified in the same manner as in embodiment 3 to obtain an antibody recognizing the C-terminus peptide of amyloid beta (1-42). This antibody was named 1C3.

[0076]

Embodiment 6

Confirmation of Reactivity of 1C3 Antibody by Immune-precipitation Method:

In order to confirm that the 1C3 antibody obtained in embodiment 5 recognizes the C-terminus peptide of the amyloid beta (1-42), the 1C3 antibody was subjected to immune-precipitation against each peptide of amyloid beta (1-40), amyloid beta (1-41), amyloid beta (1-42), and amyloid beta (1-43) according to a common method (for example, "Molecular Biology Basic Experimentation" Nankodo Co., Ltd.). 6E10 antibody (manufactured by Signet Laboratories, Inc.) was used for detection with a final blotting.

[0077]

The results of the immune precipitation were shown in FIG. 2. It was confirmed that the antibody 1C3 specifically recognizes amyloid beta (1-42), but does not recognize amyloid beta (1-40), amyloid beta (1-41), and amyloid beta (1-43).

[0078]

Embodiment 7

Preparation of Antibody Recognizing N-terminus Peptide of Amyloid beta:

A BALB/c mouse was immunized by using the bound substance of the peptide (e) and thyroglobulin obtained in embodiment 2 as an antigen for immunization. After immunization four times, the mouse was further immunized twice using the bound substance of the peptide (f) and thyroglobulin. Spleen monocyte cells of the immunized mouse and a fusion partner, X63-Ag8-653, were subjected to polyethylene glycol-mediated cell fusion and hybridomas were selected using the method described in J. Immunol. 146:3721–3728. In selecting the hybridomas, cells that react with immobilized peptide (e) and peptide (f) were selected.

[0079]

The cells selected by the above procedure were purified in the same manner as in embodiment 3 to obtain an antibody recognizing the N-terminus peptide of amyloid beta. This antibody was named 82E1.

[0080]

Embodiment 8

Confirmation of Specificity of 82E1 Antibody by Western Blotting:

Next, in order to confirm that the 82E1 antibody obtained in embodiment 7 recognizes the N-terminus peptide of the amyloid beta, the 82E1 antibody was subjected to western blotting against each peptide of amyloid beta (1-40), amyloid beta (2-40), and amyloid beta (3-40) according to a common western blotting method (for example, "Molecular Biology Basic Experimentation" Nankodo Co., Ltd.). For comparison, Chinese hamster cells in which amyloid beta precursor proteins (APP) were forcibly exhibited were homogenized with a buffer solution containing 1% Triton and centrifuged to obtain a supernatant liquid, which was subjected to western blotting in the same manner as above. This supernatant contained, in addition to APP, amyloid beta and betaC terminal fragments (betaCTF) cut from APP at the beta site. In addition, for comparison with a conventional antibody, 6E10 antibody (manufactured by Signet Laboratories, Inc.) which is reported to recognize the N-terminus peptide of amyloid beta was subjected to western blotting using the same sample as above.

[0081]

The results of western blotting are shown in FIGS. 3 and 4. It was confirmed from FIG. 3 that the antibody 82E1 does not react with amyloid beta (2-40) and amyloid beta (3-40), but recognizes only amyloid beta (1-40). On the other hand, 6E10 antibody was confirmed to be unreactive with amyloid beta (1-40), but to recognize amyloid beta (2-40) and amyloid beta (3-40). In addition, based on FIG. 4, it was confirmed that 82E1 monoclonal antibody does not react with APP, but recognizes only amyloid beta and betaCTF. On the other hand, 6E10 antibody was confirmed to recognize APP.

[0082]

Embodiment 9

Preparation of Bound Substance of Antibody Recognizing N-terminus Peptide of Amyloid beta (82E1 antibody) and HRP:

The bound substance of the 82E1 antibody obtained in embodiment 7 and HRP was prepared as follows. A necessary amount of HRP was dissolved in distilled water, oxidized with NaIO$_4$, and dialyzed with a 1 mM acetic acid buffer solution (pH 4.4) overnight. 2 mg of 82E1 antibody was also dialyzed with a 0.1 M carboxylic acid buffer solution (pH 9.5) overnight. The dialyzed 82E1 antibody and HRP were mixed at a ratio of 0.4 mg of HRP to 1 mg of the antibody and reacted at room temperature for two hours. NaBH$_4$ was added to the reaction mixture. After reaction in ice for two hours, the resulting reaction product was dialyzed with PBS overnight. The reaction product was further subjected to gel permeation to obtain a bound substance of the 82E1 antibody and HRP.

[0083]

Embodiment 10

Construction of Sandwich ELISA Method:

The sandwich ELISA method using the antibodies obtained in the above embodiments was constructed as follows. 20 μg/ml 1A10 antibody or 1C3 antibody was added to 96-well plates for ELISA analysis in an amount of 100 μl each. The antibodies were incubated at 4 °C overnight, followed by blocking using a 1% BSA/PBS/NaN$_3$ solution, to obtain a plate for the sandwich ELISA method. The bound substance of the 82E1 antibody obtained in embodiment 6 and HRP was used as the labeled antibody.

[0084]

The peptides of the synthetic amyloid beta (1-40) and synthetic amyloid beta (1-42) were respectively assayed using the ELISA plate and labeled antibody. The resulting standard curves are shown in FIG. 5, A is a result of assaying the synthetic amyloid beta (1-40) using the combination of the 1A10 antibody and HRP labeled 82E1 antibody and B is a result of assaying the synthetic amyloid beta (1-42) using the combination of the 1C3 antibody and HRP labeled 82E1 antibody. Both results exhibited an excellent concentration-depending linearity.

[Industrial Applicability]

[0085]

The antibody which recognizes the N-terminus peptide of an amyloid beta of the present invention recognizes the amyloid beta, but does not recognize an amyloid beta precursor protein. Then, the amyloid beta assay kit using this can precisely assay the amyloid beta (1-42) or amyloid beta (1-40) possessing a complete length ,unlike amyloid beta assay kits heretofore made commercially available.

[0086]

Therefore, the amyloid beta assay kit can be used for diagnosing Alzheimer's disease in which the amyloid beta is involved and for research on the mechanism and the like of the disease occurrence. One specific example that can be given is a search of beta secretase inhibitors.

[Brief Description of the Drawings]

[0087]

[FIG. 1] FIG. 1 is a drawing showing the specificity of the 1A10 antibody by western blotting.

[FIG. 2] FIG. 2 is a drawing showing the specificity of the 1C3 antibody by immunological precipitation.

[FIG. 3] FIG. 3 is a drawing showing the specificity of the 82E1 antibody by western blotting (A: 82E1 antibody, B; 6E10 antibody).

[FIG. 4] FIG. 4 is a drawing showing the specificity of the 82E1 antibody by western blotting (A: 82E1 antibody, B; 6E10 antibody).

[FIG. 5] FIG. 5 is a drawing showing the results of standard curves obtained by assaying a synthetic amyloid beta using an ELISA kit prepared in embodiment 10 (A shows the result of assaying a synthetic amyloid P (1-40) using a combination of the 1A10 antibody and HRP labeled 82E1 antibody and B shows the result of assaying a synthetic amyloid beta (1-42) using a combination of the 1C3 antibody and HRP labeled 82E1 antibody).

[Sequence Listing]

                           SEQUENCE LISTING

<110> Immuno-Biological Laboratories Co., Ltd.

<120> Monoclonal antibody and its utilization

<130> 0310131

<160> 6

<170> PatentIn version 3.1

<210> 1
<211> 16
<212> PRT
<213> Homo sapiens

<400> 1

Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys
1   5   10   15

<210> 2
<211> 5
<212> PRT
<213> Homo sapiens

<400> 2

Asp Ala Glu Phe Arg
1   5

<210> 3
<211> 6
<212> PRT
<213> Homo sapiens

<400> 3

Met Val Gly Gly Val Val
1   5

<210> 4
<211> 5
<212> PRT
<213> Homo sapiens

<400> 4

Gly Val Val Ile Ala
1   5

<210> 5
<211> 40
<212> PRT
<213> Homo sapiens

<400> 5

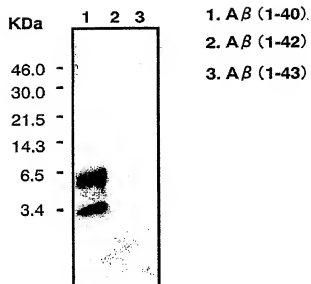Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys
1   5   10   15

Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile
            20   25   30

Gly Leu Met Val Gly Gly Val Val
        35   40

<210> 6
<211> 42
<212> PRT
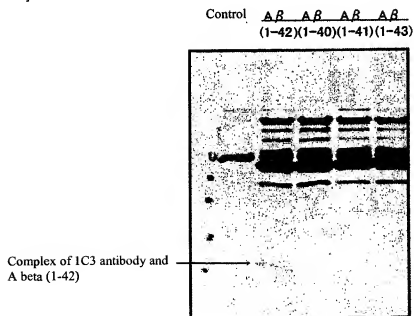<213> Homo sapiens

<400> 6

Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys
1   5   10   15

Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile
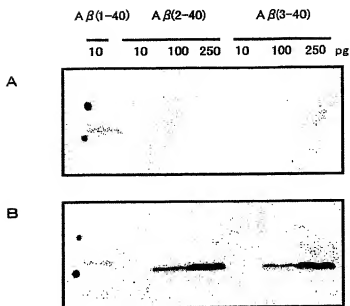   20   25   30

Gly Leu Met Val Gly Gly Val Val Ile Ala
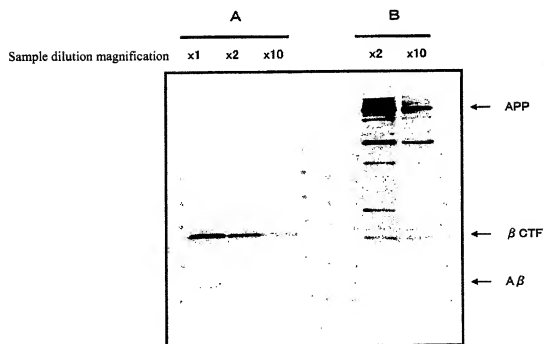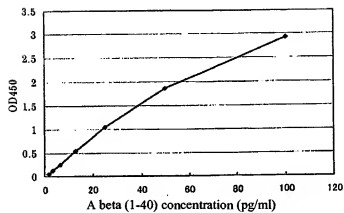   35   40

[Document Title] Drawings
[FIG. 1]

KDa   1 2 3   1. Aβ (1-40).

46.0 -   2. Aβ (1-42)
30.0 -
21.5 -   3. Aβ (1-43)
14.3 -
6.5 -
3.4 -

[FIG. 2]

Control   Aβ   Aβ   Aβ   Aβ
         (1-42)(1-40)(1-41)(1-43)

Complex of 1C3 antibody and
A beta (1-42)

[FIG. 3]

[FIG. 4]

[FIG. 5]

A

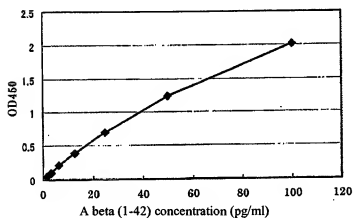A beta (1-40) concentration (pg/ml)

B

A beta (1-42) concentration (pg/ml)

[Document Title] Abstract
[Abstract]

[Problem] To provide a system for precisely assaying amyloid beta (1-40) and amyloid beta (1-42) which completely possess the entire length.
[Means for Solving] A monoclonal antibody that can recognize the N-terminus peptide of an amyloid beta but does not recognize the amyloid beta precursor protein, and an amyloid beta assay kit that uses this.
[Selected Drawing] FIG. 4

Applicant History Information

ID No.   [399032282]

1. Modification date: May 24, 1999
[Reason for Modification]   New registration
Address   1091-1 Naka Aza Higashida, Fujioka, Gunma Prefecture
Name   Immuno-Biological Laboratories, Co., Ltd.